Laboratory Procedure Manual

Analyte: **Human Papillomavirus (HPV) Typing Assay**

Matrix: **Vaginal Swab**

Method: **Roche Reverse Line-Blot HPV Typing Assay (Prototype)**

Method No.:

Revised:

as performed by: *Viral Exanthems and Herpes Virus Branch*
Division of Viral and Rickettsial Diseases
National Center for Infectious Diseases

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Important Information for Users

The National Center for Infectious Diseases/CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 2003–2004 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
l37_c	LBDH4	HPV PCR band
	LBDGL	Globin PCR band
	LBDH06	HPV type 06
	LBDH11	HPV type 11
	LBDH16	HPV type 16
	LBDH18	HPV type 18
	LBDH26	HPV type 26
	LBDH31	HPV type 31
	LBDH33	HPV type 33
	LBDH35	HPV type 35
	LBDH39	HPV type 39
	LBDH40	HPV type 40
	LBDH42	HPV type 42
	LBDH45	HPV type 45
	LBDH51	HPV type 51
	LBDH52	HPV type 52
	LBDH53	HPV type 53
	LBDH54	HPV type 54
	LBDH55	HPV type 55
	LBDH56	HPV type 56
	LBDH57	HPV type 57

l37_c	LBDH57	HPV type 57
	LBDH58	HPV type 58
	LBDH59	HPV type 59
	LBDH61	HPV type 61
	LBDH62	HPV type 62
	LBDH64	HPV type 64
	LBDH66	HPV type 66
	LBDH67	HPV type 67
	LBDH68	HPV type 68
	LBDH69	HPV type 69
	LBDH70	HPV type 70
	LBDH71	HPV type 71
	LBDH72	HPV type 72
	LBDH73	HPV type 73
	LBDH81	HPV type 81
	LBDH82	HPV type 82
	LBDH83	HPV type 83
	LBDH84	HPV type 84
	LBDH89	HPV type 89
	LBDHPI	HPV type IS39
	LBX16V	HPV type 16 variant
	LBXNST	other HPV strip types

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The assay uses amplification of target DNA by the Polymerase Chain Reaction (PCR) and nucleic acid hybridization for the detection of 37 anogenital HPV DNA genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39, and 89) in cervical cells. In addition, other types are sometimes amplified by the primer mix; if these are identified by a band on an agarose gel and a negative Roche panel, the type is confirmed by standard Sanger sequencing.

Data from this assay show the prevalence of various HPV types throughout the United States; this information is of interest in light of vaccines which are in clinical trials.

2. SAFETY PRECAUTIONS

HANDLE ALL ASSAY SPECIMENS AND DISPOSED MATERIALS AS IF CAPABLE OF TRANSMITTING INFECTIOUS AGENTS.

A. Patient specimens should be handled at the BSL 2 safety level as recommended for any potentially infectious human serum or blood specimen in the CDC-NIH manual, Biosafety in Microbiological and Biomedical Laboratories, 1984, pages 12-16.

B. Do not pipette by mouth.

C. Wear disposable powder-free gloves while handling reagents or specimens. Wash hands thoroughly after performing the test.

D. All materials used in this assay, including reagents and specimens, should be disposed of in a manner that will inactivate infectious agents.

Solid Wastes: Autoclave.

Liquid Wastes: Add sodium hypochlorite to a final concentration of 1.0% (1:5 dilution of household bleach). Allow 30 minutes for decontamination before disposal.

Spills: Non-base-containing spills should be wiped thoroughly with a 5% sodium hypochlorite solution (full-strength household bleach). Base-containing spills should be neutralized, wiped dry, and then the spill areas should be wiped with a 5% sodium hypochlorite solution. The wiped area should be covered with absorbent material, saturated with a 5% sodium hypochlorite solution and allowed to stand for at least 10 minutes. A glass or plastic cover or tray can be used to reduce exposure to fumes. All wiping materials should be treated as hazardous waste.

E. Handling Precautions

Performing the assay outside the time and temperature ranges provided may produce invalid results. Assays not failing within the established time and temperature ranges must be repeated.

Do not use the reagents beyond the expiration date on the outer box label.

(1) The Digene HPV Test Procedure, Quality Control, and the Interpretation of Specimen Results must be followed closely to obtain reliable test results.

(2) It is important to pipette the exact reagent volume indicated and to mix well after each reagent addition. Failure to do so could result in erroneous test results. Ensuring that the noted color changes occur will help confirm that these conditions have been met.

(3) These components have been tested as a unit. Do not interchange components from other sources or from different lots.

(4) Nucleic acids are very sensitive to environmental nuclease degradation. Nucleases are present on human skin and on surfaces or materials handled by humans. Clean and cover work surfaces with disposable pads and wear powder-free gloves when performing all assay steps.

(5) 40% (w/w) Dimethylformamide (DMF) is toxic, and 1.6% NaOH is in an irritant. Use caution and wear proper protective clothing when handling these chemicals. DMF must be disposed of through the hazardous chemical waste management system.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

HPV types are recorded in an Access database. These values are downloaded to an Excel file for data storage and subsequent file transport to WESTAT. In addition, printed copies of each assay, together with quality control calculations, are stored in notebooks.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

Testing for HPV DNA will be done from vaginal fluids collected using vaginal swabs. Studies have demonstrated that recovery of HPV from self-collected swabs is comparable to that from physician-collected cervical samples.

Female study participants aged 14–59 will be eligible for HPV testing using a self-administered vaginal swab. The MEC Physician will explain the soft foam vaginal swab collection technique to the study participant. After the physician goes through the written directions with the study participant, the study participant will be given the vaginal swab, a written set of directions, and directions to the bathroom. If the study participant is uncomfortable with this procedure, the physician will try to answer concerns and questions or use completion codes for refusal. A study coordinator will collect the self-administered swabs, log the study participant out of the room, escort her to the next component, and deliver specimens to the lab.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

A. Instrumentation

(1) Dry heat block 56°C ± 2°C

(2) Dry heat block 70°C ± 2°C

(3) Water bath 53°C

(4) Gemini Twin temp shaking water bath

(5) Applied Biosystems 9700 thermal cycler

(6) Invitrogen E-Gel powerbase v.4

B. Other materials

(1) Sterile disposable, polystyrene serological pipettes (5 mL, 10 mL, and 25 mL)

(2) Multichannel pipettor (capacity = 100 µL)

(3) Pipettors (capacity = 20 µL, 200 µL, and 1000 µL) with aerosol barrier or positive displacement DNA and DNase-free tips

(4) Vortex mixer

(5) Distilled or deionized water

(6) Invitrogen E-Gel 2 % agarose gels

C. Reagent Preparation

Figure 1.

	% Pipette excess	12		**No. Tubes to set up**	112
				Reaction mixture total volume	100
Master Mix	**Stock Conc.**	**Final Conc.**	**Units**	**1 X Vol.**	**Use**
H_2O				61.4	7702.016
10X buffer II	10	1	X	10	1254.4
$MgCl_2$	25	4	mM	16	2007.04
dNTP	10000	200	µM	2	250.88
PGmy09	50	1	µM	2	250.88
PGmy11	50	1	µM	2	250.88
ß-PCO_4	50	0.025	µM	0.05	6.272
ß-GH_2O	50	0.025	µM	0.05	6.272
Taq	5	0.075	U/µl	1.5	188.16
			Master Mix Sub-total	95	
			Volume of template per reaction	5	

(1) Amplify in Applied Biosystems 9700 thermal cycler, using the following program:

- 95° for 9 minutes
- 40 cycles of:
- 95° for 30 seconds
- 55° for 1 minute
- 72° for 1 minute
- 4° hold

} 40cycles

(2) Run 10 µl of each sample on an Invitrogen 2% agarose E-Gel. Perform the Roche HPV typing assay on all samples which give a band of around 450bp, which is the size of the HPV amplicon.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration Curve

No calibration curve is generated by the user as part of these assay methods.

B. Verification

Verification for this assay is not possible in the conventional manner. The investigators who read assay results are trained to analyze the positive and negative controls for each test series. Each tray of samples also includes a positive control consisting of HPV DNA from an HPV16 cell line that is

amplified with the test samples. The concentration of this sample is such that it serves as a serves as a reagent control; a negative result indicates that the entire assay must be repeated.

8. PROCEDURE OPERATING INSTRUCTIONS:

A. Roche Human Papillomavirus Typing Assay

(1) Prewarm the 1X Hybridization Solution and the 1X Wash Solution in a 53°C water bath. Always verify the temperature in the water bath with an immersion thermometer.

(2) While buffers are warming, set up detection bench (area should only be used for amplification and detection of amplified DNA). Lay blue diaper on work surface. Have reagents ready, pipettes, vacuum system and 4 typing trays (Dynal RELI SSO Typing Trays, cat# 803.00). Tray 1 should be labeled for hybridization, tray 2 for conjugate, tray 3 for washes, and tray 4 for substrate color detection.

(3) Once buffers have reached 53°C (all precipitated solids must be in solution), set up and label 0.6 ml tubes in rack – one for each amplified product to be detected. Add 40 µl of Denaturation solution (solution of EDTA, 1.6% sodium hydroxide, and thymol blue) to each tube.

(4) Denature PCR product by adding 40 µl of product to the corresponding tube containing 40 µl of Denaturation solution. Set rack aside while setting up strips.

(5) Note: There is no absolute time that samples should denature. Keep at room temperature while setting up strips (no longer than 2 hours).

(6) Using clean forceps, remove the appropriate number of HPV Typing Strips (stored in bags with desiccant, in box at room temperature). Place the strips on a piece of blotting paper (any size) and label with a waterproof marking pen. Place each strip face up in individual wells of typing tray #1 (hybridization tray).

(7) Add 3 ml of prewarmed Hybridization buffer to each well with labeled strip.

(8) Using separate plugged tip pipettes, add 75 µl of each of the denatured amplified samples to the appropriate wells. Pipette up and down several times and rock tray back and forth with each addition.

(9) Place the lid on the tray and set the tray in the 53°C water bath. The water level should cover the bottom "well" section of the tray. Place weights on the lid to hold tray in place. Incubate strips for 30 minutes while shaking steadily (approximately 60 rpm).

(10) Remove tray from water bath, tip tray at slight angle and aspirate the fluid from each well using vacuum system with glass pipette at end of tube. Wipe lid of tray with a Kimwipe to remove condensation.

(11) Quickly rinse the strips by adding 3 ml per well of (room temperature) Wash Buffer to each well. Tilt tray back and forth for several seconds and aspirate off the solution.

(12) Add 3 ml per well of 53°C heated Wash Buffer to each well and place tray back in 53°C water bath. Cover with weights and incubate for 15 minutes while shaking steadily.

(13) While tray is washing, prepare conjugate solution. The conjugate solution is a 1:333 dilution of Streptavidin Horseradish Peroxidase (P.E.) in Wash Buffer. Make appropriate amount necessary depending on the number of strips being made. Always prepare the conjugate in an adequately-sized disposable container.

B. Conjugate Solution (1:333) - Mix well by shaking gently

	1 strip	**10 strips**	**15 strips**	**20 strips**
P.E. Conjugate	15µl	105 µl	150 µl	195 µl
Ambient Wash Buffer	5 ml	35 ml	50 ml	65 ml

(1) At completion of stringent wash, remove tray from water bath and turn off water bath. Tilt tray at an angle and aspirate fluid from each well.

(2) Using forceps carefully transfer each strip to tray #2 (conjugate tray). Dispense 3 ml of conjugate solution to each well. Place tray on rocker and cover with weights. Incubate at room temperature for 30 minutes while rocking steadily.

(3) Remove tray from rocker and aspirate conjugate solution. Quickly rinse strips with 3 ml of Ambient Wash Buffer. Tilt tray back and forth several seconds. Aspirate Wash Buffer and using forceps carefully transfer each strip to tray #3 (wash tray).

(4) Add 3 ml of Ambient Wash Buffer to each well. Place tray on rocker and cover with weights. Incubate at room temperature for 10 minutes while rocking steadily.

(5) During this wash, prepare working substrate. The substrate is a mixture of Substrate A (a citrate solution containing 0.01% H_2O_2 and 0.1% Pro Clin 150 preservative) and Substrate B (0.1% 3,3', 5, 5'-tetramethylbensidine (TMB) and 40% dimethylformamide (DMF)). Prepare appropriate volume for number of strips being detected in a disposable container. Once reagents are mixed, cover container with foil to protect from direct exposure to light.

C. Substrate Solution: Mix well by shaking

x	1 strip	10 strips	15 strips	20 strips
Substrate A	4.2 ml	25.2 ml	37.8 ml	50.4 ml
Substrate B	1.2 ml	7.2 ml	10.8 ml	14.4 ml

Note: Substrate B contains 40% formamide, a hazardous chemical, which should be treated and disposed of according to hazardous materials management standards.

(1) Aspirate Wash Buffer and add 3 ml of Wash Buffer to each well. Place tray on rocker and cover with weights. Incubate at room temperature for 10 minutes while rocking steadily.

(2) Repeat step 19 one time. This will be a total of three 10-minute ambient washes.

(3) Once washes are complete, aspirate wash buffer from each well and carefully transfer strips to tray #4 (substrate tray) using forceps. Add 3 ml of Citrate Buffer and allow strips to equilibrate for approximately 5 minutes.

(4) Aspirate Citrate Buffer and add 3 ml of Substrate to each well. Place tray on rocker and incubate for 5 minutes while rocking steadily.

(5) Remove tray from rocker and remove substrate from each well with a pipette. Place substrate waste into correctly labeled hazardous materials container. Rinse each well twice with distilled water. Remove the first wash with a pipette and dispose of rinse in hazardous waste container. Second water rinse may be aspirated with vacuum.

(6) Transfer strips to tray #5 (citrate buffer tray) and add 3 ml of Citrate Buffer to each well. Place tray on rocker and cover with weights. Incubate at room temperature for 10 minutes while rocking steadily.

(7) Aspirate Citrate Buffer from each well. Using forceps carefully transfer strips to blot paper and place in 80°C oven to dry for 10 minutes.

(8) Use Roche Strip Template to read results by lining up transparency above each strip and match reference line on template with reference line on strip. Bands present that are equal or below background are considered negative.

(9) Record results on worksheet.

(10) Save strips for notebook by taping to a new piece of blot paper labeled with Roche date, Roche strip Lot #, and seal in a reaction folder (Schleicher & Schuell, item #10483064).

9. REPORTABLE RANGE OF RESULTS

Final reports express results as positive or negative for the presence of HPV DNA genotypes in the sample in the sample.

10. QUALITY CONTROL (QC) PROCEDURES

Each Linear Array strip contains two probes for high and low concentrations of globin in the sample. Globin primers are present in the PCR master mix and these will be amplified and detected along with any HPV DNA in the patient sample. All human epithelial cells contain DNA for the beta globin protein; therefore, a positive result for beta globin is an indication of adequate and detectable patient sample. Occasionally, the beta globin probes will give a negative result when a positive HPV result is present; this is because the ratio of globin primers to HPV primers is optimized for the detection of HPV DNA.

Each tray of samples includes a water blank that has been present throughout the entire processing of that group of samples; this serves as a contamination control; if it is positive, new aliquots of the whole sample group are processed again.

Each tray of samples also includes a positive control consisting of HPV DNA from an HPV16 cell line that is amplified with the test samples. The concentration of this sample is such that it serves as a serves as a reagent control; a negative result indicates that the entire assay must be repeated.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

Repeat assay using different lot numbers of reagents.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Adequate sample collection, transport, storage and processing procedures contribute to overall test performance.

Samples containing greater than 3.5% (v/v) blood have been shown to inhibit PCR amplification and may give false negative or invalid results.

Some gel contraceptives may interfere with test.

13. REFERENCE RANGES (NORMAL VALUES)

A negative result is considered normal for this type of testing.

14. CRITICAL CALL RESULTS (PANIC VALUES)

Not applicable.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

All testing is done on an aliquot of DNA extracted from a portion of the original sample. The DNA is stored at –20°C, and the remainder of the original sample is stored in Digene specimen transport medium at –86°C.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

There are no alternative methods. Test may be repeated with fresh reagents. Storage systems are monitored 24 hours/day. If problem seems lot-specific, Roche is notified.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

There are no associated critical call results for this type of testing.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

All sample leftover from testing is stored at –86°C. DNA aliquots are stored at –20°C. Sample logs with location data are kept. Samples will not be transferred to other individuals without approval from NHANES.

19. SUMMARY STATISTICS and QC GRAPHS

Qualitative assays are qualitative assays with a positive or negative result. The absorbance or reactivity values of specimens are compared with a RLU/cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.